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Two Clinical Isolates and the Toledo Strain of Cytomegalovirus Contain Endothelial Cell Tropic Variants That Are Not Present in the AD169, Towne, or Davis Strains

Luci P. MacCormac and Jane E. Grundy*

Department of Clinical Immunology, Royal Free Hospital School of Medicine, London, England

The highly fibroblast-passaged AD169, Towne, and Davis strains of cytomegalovirus (CMV) were found to have a restricted capacity to infect endothelial cells in vitro. Although such replication could be increased by a combination of low speed centrifugation and sodium butyrate treatment, the extracellular virus produced was infectious for fibroblasts but not for endothelial cells. In contrast, the low passage Toledo strain, and a low passage fibroblast-grown clinical isolate of CMV, C1F, could be continually passaged in endothelial cells, giving the strains C1FE and Toledo.E. Whilst, using the conditions described above, initial infection of endothelial cells with AD169 or C1F resulted in similar titres of extracellular virus as assayed on fibroblasts, only the virus from the C1F strain was infectious for endothelial cells. Passage of C1F in fibroblasts decreased its ability to infect endothelial cells, whilst retaining equal ability to infect fibroblasts. Although endothelial-cell-passaged cell-free C1FE virus was endothelial cell-tropic, it was still much more infectious for fibroblasts than for endothelial cells. It is concluded that the C1F and Toledo strains, but not the AD169, Towne, or Davis strains, contained endothelial cell tropic variants, which could be lost on passage through fibroblasts, but retained on passage through endothelial cells. Furthermore, virus in an ex vivo source of CMV, a blood specimen, was found to be more tropic for fibroblasts than for endothelial cells, suggesting that in vivo CMV exists as quasi strains with different cell tropism, some of which might be lost in vitro by passage in an inappropriate cell type. ***J. Med. Virol. 57:298–307, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: CMV; endothelium; virus replication; cell tropism

INTRODUCTION

Human cytomegalovirus (CMV) causes widespread infection in the general population, although such infection is not usually associated with overt symptoms of disease in immunocompetent individuals [Weller, 1971; Betts, 1982]. In the immunocompromised host, however, CMV infection is often associated with significant morbidity and mortality [Ho, 1977; Sonnabend et al., 1983; Jacobson and Mills, 1988], suggesting that the immune system plays an important role in limiting viral spread in the healthy individual. At the cellular level, viral replication is dependent on a complex, and in part obscure, series of regulatory factors associated both with the virus itself, and with the host cell in which the virus resides [Lafemina and Hayward, 1988; Albrecht et al., 1990]. Thus the type and physiology of the cell that the virus encounters may also influence the course of infection and disease. Endothelial cell involvement, in particular, has been considered to be a feature of more severe infections [Goodman and Porter, 1973; Foucar et al., 1981], and to contribute both to virus dissemination [Percivalle et al., 1993; Grefte et al., 1993, 1995; Waldman et al., 1995; Grundy et al., 1997] and to the disease process per se. For example, initial endothelial cell damage has been proposed to be a trigger for the subsequent development of the accelerated form of atherosclerosis seen in transplant recipients, a condition that is believed by some to be associated with CMV infection [Adam et al., 1987; Grattan et al., 1989; McDonald et al., 1989; Loebe et al., 1990; Grundy, 1998].

Despite evidence that endothelial cells are an important target for CMV in vivo, these cells were not found

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*Correspondence to: Dr. Jane E. Grundy, Department of Clinical Immunology, Royal Free Hospital School of Medicine, London NW3 2PF, England. E-mail: Dr Jane Grundy @jgrundy.com

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to be permissive for CMV replication in vitro [Friedman et al., 1981]. However, most in vitro studies were performed using laboratory strains of virus, such as AD169, which had been passaged many times in fibroblasts, and this preparation may have adversely affected the tropism for other cell types. Indeed, recent clinical isolates of CMV have been found to be more permissive for both leukocytes and endothelial cells than the laboratory strain AD169 [Einhorn and Ost, 1984; Ho et al., 1984; Waldman et al., 1989]. There are two main explanations for these observations; first, that extensive in vitro passage in fibroblasts selects out variants from the original virus population that are not endothelial-cell tropic, or alternatively, that the virus is altered in some way by passage in fibroblasts. Support for the latter proposal comes from recent reports, which indicate that highly passaged laboratory strains of CMV have large scale deletions of the viral genome when compared with recent clinical isolates, suggesting that in vitro passage may lead to the loss of viral genes [Cha et al., 1996].

To address whether endothelial cell tropism is a property of particular strains of CMV or could be acquired by passage of the virus in endothelial cells, the ability of various fibroblast-passaged "strains" of CMV to productively replicate in endothelial cells was compared, and we attempted to "adapt" these strains to endothelial cell growth. In addition, we compared the effect of endothelial cell versus fibroblast passage on the endothelial cell tropism of virus from clinical specimens.

MATERIALS AND METHODS

Cell Culture

Human embryonic lung fibroblasts were isolated from foetal lung tissue by trypsin digestion (500 μg/ml), and maintained in minimal essential media containing 10% foetal calf serum (fibroblast growth medium), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (GIBCO BRL, UK). Fibroblasts were used between passage 9 and 17. Human umbilical vein endothelial cells were isolated from umbilical cords by collagenase digestion (200 IU/ml, Boehringer Mannheim) and maintained in medium 199 containing 20% foetal calf serum (endothelial growth medium), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μg/ml endothelial cell growth factor (Sigma, UK) and 20 IU/ml heparin (CP Pharmaceuticals Ltd.). Culture flasks were pretreated for 3 hr with 2% gelatin, and endothelial cells were used between passage 2 and 4. All cells were negative when tested for mycoplasma using a [^{3}H]-labelled DNA probe homologous to *Mycoplasma* and *Acholeplasma* ribosomal RNA (Gen-probe, San Diego, CA).

Viral Stocks

The laboratory strains of human CMV, AD169, Davis, and Towne, were obtained from the American Type Culture Collection at passages 94, 76, and 132 respectively, and propagated for a further two to four passages in fibroblasts. The virus was harvested from the supernatant fluids of infected fibroblasts, clarified by centrifugation at 800 × *g* for 20 min, and stored at −80°C until use. Fibroblast cells harboring the clinical isolate of CMV, Toledo, at passage 7, were obtained from Dr. Stuart Starr (Philadelphia, PA). The CMV infected cells were propagated serially through uninfected fibroblasts, and the supernatant fluids were tested for the presence of virus when >80% of the cells were displaying late cytopathic effect. After four such passages, CMV was harvested from the infected cell supernatant fluids, as described above.

For clinical isolates, the buffy coat was removed from the blood of an human immunodeficiency virus-positive patient with CMV disease. The leukocytes were washed three times in Hank's buffered saline solution to remove excess ethylenediamine tetraacetic acid (EDTA), and the cells were inoculated onto fibroblasts. After a 60-min incubation period at 37°C in 5% CO_2, the leukocytes were removed and replaced with minimal essential medium containing 10% foetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The fibroblasts showed evidence of CMV infection 1 week later, and the infected cells were passaged serially onto uninfected fibroblasts, as described above. CMV was present in the supernatant fluids of the infected cells after five passages through the fibroblasts. The clinical isolate (C1F) was then harvested from the supernatant fluids of the infected cells, as described above. All viral stocks were negative when tested for mycoplasma using the [^{3}H]-labelled DNA probe homologous to *Mycoplasma* and *Acholeplasma* ribosomal RNA (Gen-probe). A second clinical isolate (C2) was obtained from the blood of a renal transplant patient undergoing a primary CMV infection. The leukocytes were inoculated onto fibroblasts, and the cells were treated, as described for C1F. However, in this case, the isolate (C2F) was still largely cell associated at passage 9 and little cell-free virus could be recovered; therefore, the infected cells were stored at −180°C until use.

Endothelial Cell Adaptation of Viral Stocks

C1F-infected fibroblasts were removed by trypsin digestion when the cells were showing >80% late cytopathic effect. At this stage (C1F, passage 2, $C1F_2$), the virus was still cell associated. The infected cells (approximately 3×10^5 cells) were freeze-thawed and resuspended in 3 ml of minimal essential medium containing 4% foetal calf serum and inoculated onto endothelial cells growing in a 75-cm^2 tissue culture flask. After an incubation period of 120 min, the cells were removed and 10 ml of endothelial growth medium containing 10 mM sodium butyrate was added. The drug was removed 48 hr later and replaced with endothelial growth medium. The endothelial cells were passaged serially until a late cytopathic effect developed in >80% of the cells, after which the growth medium was replaced with medium lacking heparin, and the supernatant harvested daily. The supernatants (termed

$C1F_2E_n$, where n = the number of passages in endothelial cells) were clarified as above, and aliquots stored at −80°C until use. In addition, 3 ml of clarified supernatants from C1F- (passage 5, $C1F_5$) or Toledo- (passage 11) infected fibroblasts were inoculated onto the endothelial cells by centrifugation at 1,500 × *g* for 60 min at 22°C. After a further 60-min incubation period at 37°C in 5% CO_2, the cells were treated with 10 mM sodium butyrate as above. Supernatants from both sources of inoculated endothelial cells contained virus after two to four passages (termed $C1F_5E_n$ and Toledo.E_n, respectively). To obtain the isolate C2E, leukocytes from the blood of a renal transplant patient undergoing a primary CMV infection were inoculated directly onto endothelial cells in the absence of prior fibroblast isolation. The endothelial cells were passaged subsequently as required, although in this case, no sodium butyrate was added. As the isolate was still largely cell-associated at passage 9, the cells were stored at −180°C until use.

Viral Infection of Cell Monolayers

Cell monolayers were infected with CMV the day after initial plating. Cells were seeded in six well plates at a concentration of 5×10^5/well, washed 3× in Hank's balanced salt solution, and inoculated with 1 ml of viral supernatant. Where indicated, the plates were centrifuged at 1,500 × *g* for 60 min at 22°C, followed by a further 60-min incubation at 37°C in 5% CO_2. The supernatant was removed, the cells were washed 3× in Hank's balanced salt solution, and fresh endothelial growth medium was added. For some experiments, 10 mM of sodium butyrate was added to the medium, as indicated.

Monoclonal Antibodies

The antibody E13, which recognises CMV immediate early antigens 1 and 2, was used at a concentration of 7 μg/ml (Biosoft, TCS, UK). The antibodies p63/27 and 7.17, which recognise the CMV immediate early antigen 1 and the glycoprotein B late antigen, respectively, were used at a dilution of 1/100 of ascitic fluid (Dr. W. Britt, Birmingham, AL). An affinity-purified $F(ab)_2$ fraction of a fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse antibody (Sigma) was used at a dilution of 1/80. Affinity-purified Fc fragments of human IgG (Calbiochem, UK) were used at a concentration of 10 μg/ml.

Immunofluorescent Staining

CMV-infected endothelial cells or fibroblasts were grown in six-well plates and the cells were removed by trypsin (GIBCO) digestion before being used to make cytocentrifuge preparations. The cells were fixed in acetone for 20 min at 4°C and the slides stored at −20°C until use. Immunofluorescent staining for the presence of the CMV immediate early 1 or gB antigens was performed using the antibodies 63/27 or 7-17 respectively as described elsewhere [MacCormac and Grundy, 1996].

Detection of CMV Immediate Early Antigen by Flow Cytometry

CMV-infected endothelial cells or fibroblasts were grown in 6 well plates and the cells were removed by trypsin digestion (GIBCO). The cells were fixed in acetone and stained for the presence of CMV immediate early 1 and 2 antigens using the E13 antibody as described elsewhere [MacCormac and Grundy, 1996]. The cells were analysed using a Facscan IV flow cytometer (Beckton-Dickinson, Oxford, UK) and the data processed using the Consort 30 and Lysis II software programmes.

Determination of CMV Titre in Supernatant Fluids

The titre of CMV present in supernatant fluids was determined using a conventional plaque assay on fibroblast monolayers as described elsewhere [Roy and Grundy, 1992].

RESULTS

Conditions for the Infection of Endothelial Cells With the Laboratory Strain of CMV, AD169

The laboratory strain of CMV, AD169, was used to optimise the conditions under which highly passaged, fibroblast grown stocks of CMV could infect endothelial cells. As our initial experiments indicated that AD169 had a limited capacity to infect this cell type (<3% at a multiplicity of infection [MOI] of 5, data not shown), we tested the effect of the short chain fatty acid sodium butyrate, and the effect of low speed centrifugation, on the ability of this viral strain to infect endothelial cells. Endothelial cells, which had been inoculated with AD169 either with or without a low speed centrifugation step, were treated for various periods of time with 10 mM of sodium butyrate, and the percentage of cells expressing CMV immediate early antigen was determined by flow cytometry (Fig. 1). In the absence of centrifugal inoculation, endothelial cells that had been treated with sodium butyrate, both before and after viral inoculation, showed a marginal increase in the percentage of immediate early antigen positive cells, with the maximal effect (15%) occurring when the cells had been treated with the drug for 2 days postinoculation (Fig. 1). The addition of the drug to the endothelial cells for periods longer than 2 days resulted in substantial cell death (data not shown).

A more marked increase in the percentage of cells expressing CMV immediate early antigen occurred when the virus had been inoculated onto the endothelial cells by centrifugation (Fig. 1). In all cases, the percentage of CMV immediate early antigen-positive cells was increased in relation to those cells in which the virus had not been centrifugally inoculated, but which had been treated with sodium butyrate for the same length of time. Once again, treatment of the cells for 2 days postinoculation resulted in maximal expression of the viral antigen, although in this case, the ad-

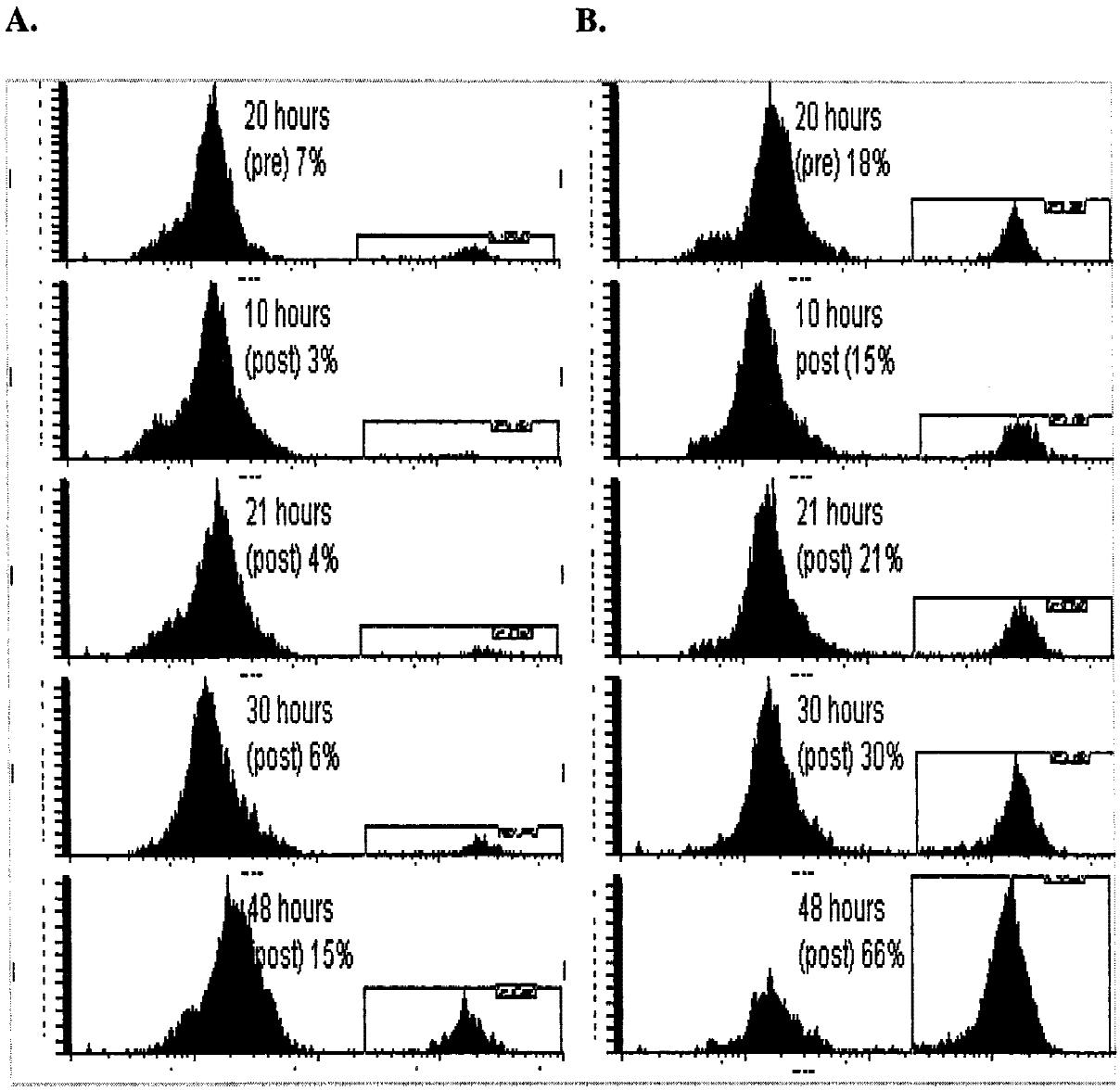

Fig. 1. The effect of sodium butyrate treatment and centrifugal enhancement on the expression of cytomegalovirus (CMV) immediate early antigen in endothelial cells. Endothelial cells were inoculated with CMV strain AD169 (multiplicity of infection = 5) and treated for various periods, before (pre) or after (post) inoculation, with 10 mM sodium butyrate. The percentage of CMV immediate early antigen expression was determined at 5 days postinoculation by flow cytometry. In (**A**), the virus was added to the cells in the absence of a low speed centrifugation step, whilst in (**B**), the virus was inoculated for 1 hr at 1,500 × *g*. The percentages relate to the number of CMV immediate early antigen positive cells that are depicted within the right-hand box.

ditional centrifugation step increased the number of CMV immediate early antigen positive cells to 66% (Fig. 1). Thus, the consistent increase in the number of cells that expressed CMV immediate early antigen after centrifugal inoculation led to the use of this process for the inoculation of endothelial cells in all subsequent experiments. Using the optimal conditions of centrifugal inoculation and sodium butyrate treatment (for 2 days after virus inoculation), the use of higher titres of AD169 (up to an MOI of 20) only increased the percentage of cells expressing CMV immediate early antigen to a maximum of 80% (data not shown).

Comparison of the Ability of the Laboratory Strain AD169 or a Low Passage Clinical Isolate of CMV, C1F, To Be Propagated in Endothelial Cells

We compared the ability of the laboratory strain AD169 to be propagated in endothelial cells with that of a recent clinical isolate of CMV. The clinical isolate was obtained by inoculation of a blood specimen containing CMV onto fibroblasts, and the CMV strain thus isolated was designated C1F. The virus at this initial stage was still cell associated, and was therefore pas-

TABLE I. Comparison of the Ability of a Low Passage Clinical Isolate or a High Passage Laboratory Strain of CMV to Infect Endothelial Cells

Passage number	No sodium butyrate		Plus sodium butyrate	
	AD169	C1F5	AD169	C1F5
0[a]	<1%[a]	<1%[a]	20%[a]	20%[a]
1[b]	nd[b]	nd[b]	nd[b]	nd[b]
2	1%	5%	2%	9%
3	1%	8%	2%	23%
4	1%	18%	1%	56%
5	1%	61%	1%	99%
6	1%	99%	1%	nd

CMV, cytomegalovirus; nd, not done.
Endothelial cells were centrifugally inoculated with the laboratory strain AD159 (1×10^6 pfu/ml), or a clinical isolate (4×10^5 pfu/ml) which had been passaged through fibroblasts five times ($C1F_5$). The endothelial cells were treated with 10 mM of sodium butyrate for 2 days, or left untreated. The percentage of cells expressing CMV immediate early antigen was determined by flow cytometry at each sequential passage.
[a]Visual determination of CMV antigen expression using cytospin preparations of the cells, 5 days postinoculation.
[b]Too few cells to determine CMV antigen expression using flow cytometry.

saged sequentially by plating the C1F-infected cells onto fresh, uninfected fibroblasts. The number of infected fibroblasts was expanded in this way until the virus could be detected in the supernatant fluids of the infected cells (which occurred after five such passages), and the cell-free virus stock was termed $C1F_5$. Endothelial cells were then inoculated centrifugally with $C1F_5$, and either treated with sodium butyrate for 2 days, or left untreated. CMV strain AD169 was inoculated onto the endothelial cells in parallel, using the same conditions as for $C1F_5$. When cytocentrifuge preparations of these endothelial cells were analysed 5 days after the removal of sodium butyrate, approximately 20% of the sodium butyrate-treated cells inoculated with either $C1F_5$ or AD169 were positive for immediate early antigen expression, whilst approximately 15% were positive for gB expression. Neither antigen could be detected in cells which had been inoculated with either virus strain, but which had not been treated with sodium butyrate. All inoculated cells were subsequently maintained by serial propagation (in the absence of sodium butyrate treatment), and an aliquot of these cells was analysed by flow cytometry for the expression of CMV immediate early antigen, after each passage. This process was continued for either 2 months (approximately six to eight passages), or until all cells showed characteristic late cytopathic effect. Treatment with sodium butyrate after each cell passage was toxic to the endothelial cells, therefore this treatment was only used directly after the initial viral inoculation.

The results are shown in Table I. Continual passage of endothelial cells that had been inoculated with the clinical isolate $C1F_5$ led to a progressive increase, after each passage, in the percentage of cells expressing CMV immediate early antigen. This result was found for both the endothelial cells that initially had been treated with sodium butyrate, and those cells that had received no drug treatment (Table I). The percentage of immediate early antigen positive cells was always greater, however, with the endothelial cells. In contrast, attempts to propagate the AD169 strain of virus serially in the endothelial cells were unsuccessful. Even after six passages, CMV immediate early antigen was undetectable in endothelial cells inoculated with AD169, whilst virtually 100% of the cells inoculated with $C1F_5$ were antigen positive by this stage (Table I). The cells inoculated with AD169 were passaged for a further month, but remained negative for CMV immediate early antigen expression (data not shown). Thus, despite the use of sodium butyrate treatment to initiate a cycle of replication of CMV strain AD169 in a proportion of endothelial cells, this virus strain failed to spread to surrounding uninfected endothelial cells, and could not be propagated in this cell type. In contrast, the clinical strain $C1F_5$ spread progressively in endothelial cells and eventually infected all available cells, even though the initial titre of $C1F_5$ used was lower than that of AD169 (4×10^5 and 1×10^6 pfu/ml when titrated on fibroblasts, respectively).

Comparison of the Ability of AD169 and C1F To Produce Infectious Virus in Endothelial Cells

To see whether the difference in the ability of CMV strains C1F and AD169 to spread to uninfected endothelial cells was due to an abortive cycle of replication in the case of the AD169 strain, the amount of infectious virus secreted into the supernatant fluids by endothelial cells that had been inoculated with the two virus strains was compared. As the original inoculum of C1F, which had been propagated five times through fibroblasts ($C1F_5$), contained less virus than did the AD169 stocks of CMV (4×10^5 and 1×10^6 pfu/ml respectively), the clinical isolate was propagated a further three times through fibroblasts to increase the viral titre to that of the AD169 stock. This stock was thus termed $C1F_8$. Endothelial cells were then infected with AD169, $C1F_5$, or $C1F_8$, and the cells were treated subsequently with sodium butyrate for 2 days. The supernatant fluids were removed at various times after inoculation, and the titre of infectious virus present in these cell-free fluids was estimated using a standard plaque assay on fibroblasts. To confirm the lack of ability of CMV strain AD169 to spread from infected to

TABLE II. Comparison of the Ability of a Low Passage Clinical Isolate or a High Passage Laboratory Strain of CMV to Secrete Virus Into the Supernatant Fluids of Infected Endothelial Cells

	No sodium butyrate			Plus sodium butyrate		
Day[a]	AD169	C1F5	C1F8	AD169	C1F5	C1F8
8	2.4×10^3 ±0.1	1.8×10^3 ±0.1	0.7×10^3 ±0.1	0.8×10^4 ±0.05	0.5×10^4 ±0.05	0.5×10^4 ±0.05
10	2.4×10^3 ±0.1	2.5×10^3 ±0.3	2.7×10^3 ±0.2	1.4×10^4 ±0.09	0.9×10^4 ±0.04	1.2×10^4 ±0.09
12	3.3×10^3 ±0.2	1.9×10^3 ±0.3	2.7×10^3 ±0.5	1.8×10^4 ±0.13	1.0×10^4 0.14	0.97×10^4 0.01
15	2.9×10^3 ±0.6	1.4×10^3 ±0.5	4.3×10^3 ±0.5	$\pm 3.4 \times 10^4$ ±0.3	$\pm 3.2 \times 10^4$ ±0.04	3.9×10^4 ±0.15

Endothelial cells were infected with the laboratory strain AD169, or a clinical isolate of CMV which had been passaged either five ($C1F_5$) or eight ($C1F_8$) times through fibroblasts. The cells were centrifugally inoculated with either 1×10^6 pfu/ml (AD169 & $C1F_8$), or 4×10^5 pfu/ml ($C1F_5$), and then treated with 10 mM sodium butyrate for 2 days, or left untreated. The supernatants from the infected cells were harvested at various time points and the cell-free viral titre was determined using a standard fibroblast plaque assay. Values are in pfu/ml ± SD.
[a]Day refers to the day postinoculation.

uninfected endothelial cells in the current experiment, the inoculated cells were propagated once, left for a further 3 weeks, and then analysed for CMV immediate early antigen expression using flow cytometric techniques.

The results indicated that infectious virus was secreted into the supernatant fluids of the inoculated endothelial cells for both strain AD169 and strain C1F (stocks $C1F_5$ and $C1F_8$) for at least 2 weeks following viral inoculation (Table II). Furthermore, comparable titres of virus were recovered from the supernatant fluids of endothelial cells infected with AD169, $C1F_5$, or $C1F_8$ (Table II). Sodium butyrate treatment increased the titre of extracellular virus for all three virus preparations (Table II). In accordance with the findings in the previous section, immunofluorescent staining of the endothelial cells 5 days after the removal of sodium butyrate revealed no marked differences in the percentages of cells expressing CMV immediate early antigen when strain AD169 was compared with C1F (stocks $C1F_5$ or $C1F_8$). In all cases, approximately 15–20% of the cells stained for the viral antigen after sodium butyrate treatment. However, after these cells had been propagated once, then left for a further 3-week incubation period (total incubation period of 5 weeks), only sodium butyrate-treated endothelial cells that had been inoculated with the clinical stocks $C1F_5$ or $C1F_8$, but not AD169, expressed CMV immediate early antigen (94%, 26%, and <2%, respectively). This result confirms that, during this experiment, CMV strain AD169 was unable to spread to the surrounding uninfected endothelial cells, as was found previously. In addition, the data show that substantially more cells inoculated with the lower fibroblast-passaged clinical isolate ($C1F_5$) were antigen positive when compared with the higher fibroblast-passaged ($C1F_8$) virus stock, even though the former stock was of a lower viral titre than the latter. This result suggests that passage of clinical isolates in fibroblasts might decrease their ability to spread from infected to uninfected endothelial cells.

The results thus showed that sodium butyrate treatment of the endothelial cells allowed at least one complete cycle of CMV replication to occur in a proportion of cells infected with AD169 and C1F, but that only virus secreted from C1F-infected cells could subsequently spread to uninfected endothelial cells. It is concluded that the inability of strain AD169 to spread from infected to uninfected endothelial cells was not due to the lack of production of infectious extracellular virus in this cell type.

Comparison of Propagation of a Clinical Isolate of CMV in Endothelial Cells or Fibroblasts on Its Ability to Infect These Two Cell Types

The results presented above suggested that continual fibroblast propagation of the clinical isolate of CMV, strain C1F, reduced its capacity to infect endothelial cells. Conversely, continual endothelial cell propagation of this viral strain appeared to enhance its capacity to infect endothelial cells. To study this effect further, two "stocks" of the clinical isolate C1F were produced. The first stock was propagated solely in fibroblasts for seven passages, to yield the stock $C1F_7$. The second stock was passaged a total of two times in fibroblasts followed by five passages in endothelial cells, and termed $C1F_2E_5$. Thus, both $C1F_7$ and $C1F_2E_5$ had been propagated a total of seven times. Both stocks had similar titres of extracellular virus (1.5×10^6 pfu/ml) when assayed on fibroblasts. Serial dilutions of these viral stocks were then added to both fibroblasts and endothelial cells, and the percentage of cells expressing CMV immediate early antigen determined by flow cytometry, 5 days after the sodium butyrate had been removed.

The endothelial cell-"adapted" clinical isolate $C1F_2E_5$ showed a greater ability to infect the endothelial cells than the matched fibroblast-passaged clinical isolate $C1F_7$, at all dilutions tested (Table III). Sodium butyrate treatment did not further increase the ability of $C1F_2E_5$ to replicate in the endothelial cells, whereas minimal infection occurred when $C1F_7$ was added to the endothelial cells in the absence of sodium butyrate treatment (Table III). In contrast, there was little difference in the ability of $C1F_7$ or $C1F_2E_5$ to infect fibroblasts (Table III). Both virus stocks were much more

TABLE III. Ability of a Clinical Isolate Grown Through Two Different Cell Types to Infect Either Fibroblasts or Endothelial Cells

Dilution of virus stock	Virus inoculated onto: Fibroblasts (no sodium butyrate)		Endothelial cells: No sodium butyrate		Endothelial cells: Plus sodium butyrate	
	$C1F_7$	$C1F_2E_5$	$C1F_7$	$C1F_2E_5$	$C1F_7$	$C1F_2E_5$
Neat	nd	nd	5%	76%	31%	72%
1/5	nd	nd	4%	77%	12%	71%
1/10	nd	nd	3%	61%	6%	45%
1/20	nd	nd	3%	32%	3%	31%
1/40	nd	nd	2%	29%	2%	21%
1/80	94%	87%	nd	nd	nd	nd
1/160	92%	85%	nd	nd	nd	nd

nd, not done.
Serial dilutions of a clinical isolate of cytomegalovirus (CMV) (starting concentration of 5×10^5 pfu/ml) that had been fibroblast propagated (C1F) or endothelial-cell propagated (C1FE) were inoculated onto fibroblasts or centrifugally inoculated onto endothelial cells. The endothelial cells were either treated with 10 mM of sodium butyrate for 2 days or left untreated. The percentage of cells that were positive for CMV immediate early antigen was determined at 4 days postinfection by flow cytometry.

permissive for fibroblasts than endothelial cells. It was concluded that although both the fibroblast-propagated and the endothelial-cell-propagated clinical isolates were equally able to infect fibroblasts, only virus that had been propagated serially in endothelial cells showed a propensity to infect endothelial cells.

Ability of Other Fibroblast-Propagated Strains of CMV To Infect Endothelial Cells

The above results suggested that continual passage of CMV through fibroblasts resulted in a reduction in the efficacy of the virus to infect endothelial cells, but not fibroblasts. We therefore tested the ability of three other fibroblast-propagated strains of CMV to infect endothelial cells. We used the two high-passaged laboratory strains of CMV, Towne and Davis, and a low-passaged clinical isolate, Toledo. Extracellular virus stocks from these fibroblast-passaged viral strains (1×10^6 pfu/ml) were inoculated onto the endothelial cells in the presence or absence of sodium butyrate treatment. The endothelial cells were then serially passaged for 2 months (approximately six to eight passages) and the percentage of cells expressing CMV immediate early antigen was determined by flow cytometry after each passage.

Inoculation of the fibroblast-passaged Towne, Davis, and Toledo strains of virus onto endothelial cells resulted in the initial expression (5 days after the removal of sodium butyrate) of CMV immediate early antigen in 20%, 17%, and 16% of the sodium butyrate treated cells respectively, in contrast to <1% of untreated infected cells. Serial propagation of the endothelial cells that had been inoculated with either the Towne or the Davis strain of CMV, either with or without sodium butyrate treatment, failed to promote spread of the virus to the uninfected cells, even after 2 months of continual passage. In contrast, when endothelial cells infected with the Toledo strain of virus were serially propagated, there was a progressive increase in the number of cells that displayed cytomegaly (a late cytopathic effect) after each passage. After five passages, extracellular virus secreted by these Toledo-infected endothelial cells (termed Toledo.E_5), was able to infect 24% of inoculated endothelial cells without sodium butyrate treatment, and 64% with sodium butyrate treatment. After a further three passages in endothelial cells, extracellular virus (Toledo.E_8) inoculated onto endothelial cells resulted in 60–70% of cells being immediate early antigen positive in the absence of sodium butyrate treatment, and this treatment did not further increase the percentage of positive cells. These findings are similar to those described above for the clinical isolate C1F.

When the supernatant fluids harvested up to 2 weeks after the initial endothelial cell inoculation of the fibroblast-passaged virus stocks (strains Towne, Davis, and Toledo) were analysed by plaque assays in fibroblasts, sodium-butyrate-treated endothelial cells inoculated with all three strains were found to excrete titres of approximately 10^4 pfu/ml (data not shown). These results were similar to those obtained above for the fibroblast-propagated laboratory strain of CMV, AD169. Thus, although sodium butyrate treatment had a similar affect on promoting a first round replication cycle in endothelial cells initially infected with Towne, Davis, or Toledo, only Toledo was able to spread subsequently to uninfected cells. Taken together, our results suggested that highly fibroblast-passaged strains of virus (AD169, Towne, and Davis) were inherently nonpermissive for endothelial cells, whereas strains of virus that had been passaged fewer times through fibroblasts (C1F and Toledo) had an increased propensity for infecting endothelial cells. Moreover, continual propagation of these low fibroblast-passaged viral strains through endothelial cells eventually led to the production of virus that was no longer influenced by sodium butyrate treatment of the cells (C1FE and Toledo.E).

Ability of Non-In-Vitro-Passaged CMV To Infect Endothelial Cells

The studies above used strains of CMV that had first been isolated in fibroblasts, and then "adapted" for

growth in endothelial cells. Therefore, to investigate whether endothelial cell trophic virus occurs naturally in vivo, leukocytes from a renal transplant patient undergoing a primary CMV infection were inoculated directly onto endothelial cells. After a 1-hr incubation period, the leukocytes were removed, and fresh medium, which did not contain sodium butyrate, was added. The endothelial cells were observed for any morphological changes associated with late cytopathic effect, and passaged as required. At the same time, an aliquot of the leukocytes was inoculated directly onto fibroblasts, and the cells treated in a similar manner. Cytopathic effect was observed in the fibroblasts 1 week after the original inoculation (four or five foci after one passage), with 50% of the cells involved after four passages (data not shown). The endothelial cells, however, did not show any cytopathic effect until 2 months after inoculation (a single focus of infection appearing after five passages), and a further three passages were required to produce a cytopathic effect in approximately 50% of the cells. When the supernatant fluids were tested for the presence of CMV, both the fibroblast-grown isolate (C2F) and the endothelial-cell-grown isolate (C2E) appeared to be largely cell associated, even after a total of 10 passages (data not shown). Thus we concluded that an ex vivo source of CMV replicated much more efficiently in fibroblasts than endothelial cells, and that endothelial cell trophic virus could be selected for in vitro by continual amplification through endothelial cells.

DISCUSSION

This study demonstrated that laboratory strains of CMV that have been highly passaged in fibroblasts, such as AD169, Towne, and Davis, have a restricted capacity to infect endothelial cells in vitro. Similar findings have previously been reported by others [Friedman et al., 1981; Ho et al., 1984; Smiley et al., 1988]. We showed that the infectivity for endothelial cells of all three strains could be increased by treatment with sodium butyrate, either before or after virus inoculation. Two other groups have reported similar findings for the former two strains [Radsak et al., 1989; Wu et al., 1994], although in one case, treatment of the cells prior to infection failed to have an augmentative effect. The fact that sodium butyrate could enhance CMV replication in endothelial cells after virus inoculation suggested that sodium butyrate affected events subsequent to viral entry into the cell, such as the induction of a protein necessary for viral replication, or the removal of an inhibitor of viral replication. Interestingly, the stimulation of Ras and the serine/threonine kinase Raf by sodium butyrate treatment was shown to promote transcription of the herpes simplex virus genome [Frazier et al., 1996b], and the induction of these cellular proteins by retinoic acid has been shown to the increase the permissivity of neuroblastoma cells for CMV [Angulo et al., 1995].

Our study showed that the percentage of infected endothelial cells could be further increased by low speed centrifugal inoculation of the virus, a phenomenon that also increases CMV infection of fibroblasts and monocytes [Hodgkin et al., 1988; Ho et al., 1993]. Using the combination of centrifugal inoculation plus sodium butyrate treatment, optimal infectivity of endothelial cells was achieved for the AD169, Towne, and Davis strains, with the production of extracellular virus that was infectious for fibroblasts. However, although it was possible to artificially initiate one cycle of viral replication in endothelial cells using these three highly passaged (>70 passages) strains of CMV, the virus produced did not appear to be infectious for endothelial cells, and failed to spread to neighbouring uninfected cells. In contrast, the low passage (<10 passages) clinical isolates C1F and Toledo could be propagated serially in endothelial cells, with an increasing number of inoculated cells displaying CMV immediate early antigen after each passage. Interestingly, a direct comparison of inoculation of endothelial cells with strains AD169 and C1F showed a similar percentage of cells initially expressing CMV immediate early antigen or gB, and the production of similar titres of extracellular virus (as assayed on fibroblasts) in the first 2 weeks after inoculation. Our data thus suggest that endothelial cell processing of CMV does not lead to the production of endothelial-cell-tropic virus per se; of the five CMV strains studied, only C1F and Toledo could be amplified through endothelial cells to produce cell-free virus that could replicate in endothelial cells without further treatment of the cells. Such different cell tropism associated with particular strains of CMV might be important in vivo, because in the SCID-hu mouse, the Toledo strain of CMV replicated much better than the highly passaged AD169 or Towne strains [Brown et al., 1995], and the inoculation of healthy human volunteers with the Toledo strain induced clinical illness, whilst the Towne strain did not [Quinnan et al., 1984].

Our finding that the C1F strain that had been passaged eight times in fibroblasts was less infective for endothelial cells than that passaged five times, suggested that the continual passage of CMV in fibroblasts decreased its ability to infect endothelial cells. On the other hand, propagation of the C1F strain through endothelial cells produced virus that was increasingly endothelial cell tropic, but which retained the capacity to infect fibroblasts. The latter phenomenon was demonstrated by the fact that virus which had been passaged twice in fibroblasts and then five times in endothelial cells ($C1F_2E_5$) showed increased infectivity for endothelial cells when compared with the same strain that had been passaged seven times in fibroblasts ($C1F_7$), whereas both virus preparations were equally infectious for fibroblasts. There are two possible explanations for these findings. One possibility is that all virus particles in the population contained in the original blood specimen had the ability to grow in endothelial cells, but that this property was lost after in vitro passage through fibroblasts. Alternatively, the population of virus particles originally isolated from the infected patient contained endothelial-cell-tropic variants that

were lost on passage through fibroblasts, but retained on passage through endothelial cells. The latter proposal is supported by the fact that both $C1F_7$ and $C1F_2E_5$ replicated better in fibroblasts than in endothelial cells, suggesting that a larger proportion of fibroblast-tropic virus than endothelial-tropic virus was present in these preparations. The fibroblast advantage might have been because the original isolation of CMV from the clinical sample was in fibroblasts, thus selecting out fibroblast tropic virus at the initial isolation stage. However, our demonstration that the direct isolation of virus present in the second clinical specimen, C2, was much more efficient on fibroblasts than endothelial cells, suggests that CMV, as it exists in vivo, might be less endothelial cell tropic than fibroblast tropic.

The data presented here support the probable existence of "quasi-strains" in vivo that have different cell tropisms, and some of these "quasi-strains" may be lost on passage in vitro in an inappropriate cell type. The endothelial cell is an important target cell for CMV infection in vivo, and it also plays a pivotal role in virus dissemination [Grundy, Lawson, MacCormac et al., 1997]. Further studies are imperative, therefore, to determine the factors that govern the endothelial cell tropism of this virus.

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